

United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol

## SAM 406

Supplemental Assay Method for Titration of Monovalent,  
Cell Associated Marek's Disease Vaccines of Serotypes  
1, 2, or 3

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Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3

Table of Contents

1. Introduction
2. Materials
  - 2.1 Equipment/instrumentation
  - 2.2 Reagents/supplies
3. Cell cultures
4. Preparation for the test
  - 4.1 Personnel qualifications/training
  - 4.2 Preparation of equipment/instrumentation
  - 4.3 Preparation of reagents/control procedures
  - 4.4 Preparation of the sample
5. Performance of the test
  - 5.1 Preparing dilutions and inoculating plates
6. Interpretation of the test results
  - 6.1 Controls
  - 6.2 Making plaque counts and calculating titer
  - 6.3 Retests
  - 6.4 Evaluation of test results
7. Report of test results
8. Summary of revisions
9. Appendix

Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3

## 1. Introduction

This Supplemental Assay Method (SAM) describes a procedure for titrating monovalent, cell associated, Marek's Disease Vaccines of Serotypes 1, 2, or 3 in chick embryo fibroblast (CEF) cell cultures.

## 2. Materials

### 2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

- 2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)
- 2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model No. 252)
- 2.1.3 Water-jacketed incubator with a humidified  $5 \pm 1\%$  CO<sub>2</sub> atmosphere and temperature set at  $37^{\circ} \pm 1^{\circ}\text{C}$ , (Forma Scientific, Model No. 3158)
- 2.1.4 Vortex mixer (Thermolyne Maxi Mix II Model No. M37615)
- 2.1.5 Magnetic stir plate
- 2.1.6 Scissors, sterile (Roboz Model No. RS-6800)
- 2.1.7 Curved tip forceps, sterile (V. Mueller SU 2315)
- 2.1.8 Microliter pipette (Rainin Pipetman, P1000, or equivalent)
- 2.1.9 250-ml trypsinizing flask with stir bar, sterile
- 2.2.10 Erlenmeyer flask with a stirring bar, sterile
- 2.1.11 Neubauer Hemocytometer
- 2.1.12 Bunsen burner
- 2.1.13 Blunt thumb forceps, sterile

Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3

## 2.2 Reagents/supplies

All reagents and supplies must be sterile.

- 2.2.1 Tissue culture dish, 150 x 10-mm
- 2.2.2 Tissue culture dish, 100 x 20-mm
- 2.2.3 Plastic funnel covered with 4 layers of fine gauze
- 2.2.4 Polypropylene conical tube, 29 x 114-mm, sterile, 50-ml
- 2.2.5 Polypropylene centrifuge tubes, 250-ml
- 2.2.6 Roller bottles, 1000-ml
- 2.2.7 Serological pipets
- 2.2.8 60-mm gridded cell culture dish, tissue culture treated
- 2.2.9 2 dozen specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old
- 2.2.10 Fetal Bovine Serum (FBS)
- 2.2.11 L-Glutamine
- 2.2.12 Trypsin, 0.25%
- 2.2.13 Pipette tips (Rainin 0-100, 0-200, 100-1000 or equivalent)
- 2.2.14 Solutions

All solutions are filter sterilized.

### 1. Trypsin Solution (0.25%):

Rapidly thaw frozen 2.5% (10X) trypsin in warm water. Immediately make a working solution (0.25%) by aseptically diluting the 10X solution 1:10 with Dulbecco's PBS, CaCl<sub>2</sub> and Mg free.

**Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3**

**2. Growth Medium**

Medium 199 (with Earles salts)(powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth (dry Powder)	2.95 g
NaHCO <sub>3</sub>	2.5 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
HEPES	11.97 g
Fetal Bovine Serum*(gamma-irradiated)	60 ml
q.s. with distilled or deionized water	2185 ml

Adjust pH to 7.35 to 7.4 by adding NaHCO<sub>3</sub> solution.

Before use, add 1.0 ml of a 200-mM concentration of L-glutamine per 100 ml medium.

**3. Maintenance Medium**

Medium 199 (with Earles salts)(powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth (dry powder)	2.95 g
NaHCO <sub>3</sub>	2.75 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
HEPES	11.97 g
Fetal Bovine Serum* (gamma-irradiated)	10-20 ml
q.s. with distilled or deionized water	2142 ml

Adjust pH to 7.5 by adding NaHCO<sub>3</sub> solution.

Before use, add 1.0 ml of a 200-mM concentration of L-glutamine per 100 ml medium.

\*Previously tested free of extraneous agents.

**3. Cell cultures (Secondary CEF cultures are used for the titration.)**

**3.1 Primary Chick Embryo Fibroblast (CEF) preparation**

Primary CEF cultures are prepared from specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old.

**Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3**

**3.1.1** Disinfect the air cell end of the egg with 70% ethanol and break open the shell with sterile forceps. Use the forceps to open the membranes, lift out the embryo, and place it in a sterile disposable 150 x 10-mm tissue culture or petri dish. Remove and discard the heads and viscera of the embryos with sterile forceps. Wash the embryo carcass several times with growth media (without FBS or L-glutamine) to remove excess blood. Place the washed embryos in a sterile dry 100 x 10-mm tissue culture or petri dish and mince them thoroughly using sharp sterile scissors.

**3.1.2** To further wash, put the minced tissue into a trypsinizing flask containing 50 ml growth medium (without FBS or L glutamine) and a magnetic stir bar. Place the flask on a stir plate and stir with a moderate vortex for 5 minutes. Allow the cells to settle and decant (and discard) the supernatant and repeat.

**3.1.3** To trypsinize the tissues, first rinse the residual media from the cells by adding 10 ml of the 0.25% trypsin solution, and then immediately decant the trypsin solution. Next add 40 ml of the 0.25% trypsin solution to the flask and mix on a magnetic stir plate for 15 minutes. (Set stir plate to produce a moderate vortex.)

**3.1.4** Place a sterile gauze wrapped funnel into the opening of a 250-ml conical centrifuge tube. To stop the trypsinizing action on the cells, pour 2 ml of FBS through the gauze and then pour the trypsinized contents of the flask through the gauze funnel into the centrifuge tube. Bring the total volume to approximately 125 ml with growth media. Centrifuge for 10 minutes at 250 x g (1050 rpms using a Beckman J-6B centrifuge with a JS-4.2 rotor) with the temperature set at 10°C.

**3.1.5** Observe and record the volume of packed cells, and then remove the supernatant using a 25-ml pipette. Dilute the cells approximately 1:300 with growth medium. Plant 200 ml of diluted cell suspension in each 1000-ml roller bottle. Tighten the cap and incubate for 4 days in an incubator on a roller apparatus at 37° ± 1°C. After 4 days, the cell sheet should be well proliferated and ready to split.

Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3

### 3.2 Secondary CEF preparation

Secondary CEF cultures can be prepared in the following manner, or alternate methods of preparation are acceptable:

**3.2.1** Decant the medium from 1 roller bottle of primary CEFs, pipetting any remaining media with a 5-ml pipette, and add 15 ml of the 0.25% trypsin solution pre-warmed to 37°C. Rotate the bottle until the cells begin to detach, approximately 1 minute. (The proper length of time will be learned by experience. Too short a time will result in large clumps of cells in the new suspension.) Decant the trypsin and, with an open hand, strike the side of the roller bottle until most of the cells become detached. Rinse the inside of the roller bottle with 15 ml of growth medium and swirl. Pipette the suspension with the cells into an empty Erlenmeyer flask. Repeat twice more for a total of 3 rinses. Repeat the above steps separately for each additional roller bottle.

**3.2.2** Pour the combined cell suspension from the Erlenmeyer flask through a funnel covered with 4 layers of fine gauze into a second Erlenmeyer flask containing a sterile stir bar and 25 ml of growth medium for each roller bottle used. Then rinse the funnel with an additional 25 ml of medium for each roller bottle used. Thoroughly mix the cell suspension.

**3.2.3** Quantitate the cell count of the suspension by following the procedure described in **Appendix I**. Adjust the volume so that the cell concentration that forms a confluent monolayer within 18 to 24 hours. (approximately 250,000 to 350,000 cells per ml.)

**3.2.4** Plant the secondary cell suspension into the vessels to be used for the test (usually 60-mm gridded plastic tissue culture dishes). Use 4 ml of the suspension for a 60-mm dish. The cell cultures need to be incubated in a humidified incubator with the temperature set at 37°C and the atmosphere set at 5% CO<sub>2</sub> for 18 to 24 hours prior to inoculation.

Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3

#### **4. Preparation for the test**

##### **4.1 Personnel qualifications/training**

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies and Quality Assurance guidelines of the Center for Veterinary Biologics (CVB) or equivalent; and training in the operation of the necessary laboratory equipment listed in **part 2.1**.

##### **4.2 Preparation of equipment/instrumentation**

Operate all equipment/instrumentation according to manufacturers' instructions and monitor in compliance with current corresponding CVB/National Veterinary Services Laboratories (NVSL) Standard Operating Procedures (SOPs) or equivalent.

##### **4.3 Preparation of reagents/control procedures**

Prepare reference viruses in the same manner as sample preparation.

##### **4.4 Preparation of the sample**

**4.4.1** Remove 1 ampule of vaccine from the liquid nitrogen (LN<sub>2</sub>) storage container and thaw quickly by immersing in a water bath at approximately 25°C. Immediately dilute the vaccine with the manufacturer's diluent that is warmed to room temperature. Do this by gently aspirating the vaccine into a 10-ml syringe through an 18-gauge (or larger) needle, then collect approximately 5 ml of the diluent into the same syringe and mix gently. Gently express the contents of the syringe into the bottle of diluent keeping the end of the needle in the liquid by tipping the bottle. Aspirate 2 ml from the diluted vaccine, use this to rinse the ampule once, then add it back to the diluted vaccine. Gently mix the diluted vaccine by slowly inverting the bottle. This mixture constitutes a "field strength" of the vaccine equivalent to 1 dose per 0.2 ml.



**Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3**

**4.4.2** Place the diluted vaccine in an ice bath for 2 hours (gently mix every 30 minutes) prior to proceeding with the titration. Shortly before the end of the 2 hours holding period, place 8.0 ml of growth medium (at 4°C) in 2 sterile test tubes and 9.0 ml of growth medium in 1 sterile test tube (make a set of 3 tubes for each vaccine sample). Use these to make further dilutions in the titration procedure. These dilution blanks are not held in an ice bath.

**5. Performance of the test**

**5.1 Preparing dilutions and inoculating plates**

**5.1.1** Gently mix the vaccine by inverting the bottle 10 to 15 times. Collect a sample using a 2.5 ml syringe fitted with an 18-gauge needle and add 2.0 ml to the first tube with 8 ml (a 1:5 dilution). Transfer 2.0 ml of the 1:5 dilution to a second tube with 8 ml diluent to make a 1:25 dilution. Using a clean sterile 10-ml pipette, gently mix the 1:25 dilution. Then transfer 1 ml of the 1:25 dilution to the third tube with 9 ml diluent to make a final 1:250 dilution. With SB-1 virus serotype, a final dilution of 1:125 may be desired, so the third tube may contain 8 ml diluent, in which case 2 ml of vaccine is added, for a final 1:125 dilution. Other dilution schemes can also be used.

**5.1.2** Using a clean sterile pipette, gently mix the final dilution and inoculate 1.0 ml per plate into each of 5 test plates. Do this by filling a 10-ml pipette and distribute the inoculum into the 5 plates, measuring the volume with the graduations between 2 and 7 ml. This inoculum is to be added to the 4 ml of media already in each plate. Mixing must be done thoroughly but gently to prevent rupturing of the cells. Do the dilution and inoculation as prompt as possible to prevent cells from attaching to the surface of the dilution tubes (less than 2 minutes should elapse between addition of the cell suspension to a particular dilution blank and the removal of a sample for further dilution or inoculation). Swirl the test plates as soon as each vaccine sample has been inoculated.

**Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3**

**Note:** Titrate a known positive reference virus with each group of titrations. Uninoculated negative control cells are maintained to monitor the integrity of the cell culture system.

**5.1.3** Incubate the plates in a high humidity atmosphere at  $37^{\circ}\pm 1^{\circ}\text{C}$  containing  $5 \pm 1\%$   $\text{CO}_2$ .

**5.1.4** Twenty-four hours postinoculation, remove the medium from the plates and replace with 5 ml maintenance medium. The maintenance medium may be replaced after 2 or 3 days if the pH of the culture fluids becomes too acidic.

## **6. Interpretation of the test results**

### **6.1 Controls**

The titer of the known positive reference must be within the established range for the test results to be valid. The cell controls confirm validity of the test system.

### **6.2 Making plaque counts and calculating titer**

#### **6.2.1 Counting**

Incubate the plates according to **5.1.3** until the time for counting plaques. With Serotype 3, count the plaques 5 days postinoculation. With Serotype 1 or 2, count the plaques 7 days postinoculation. Use an inverted microscope (and a grid-adapted stage if plain plates have been used) to make the counts. Count all the plaques on each of the plates of the titration series. A plaque is counted as 1 regardless of size unless it has apparently arisen from 2 distinct centers.

#### **6.2.2 Calculating**

Calculate the average number of plaques per plate and multiply this value by 25\* or 50\*\* depending on the dilution used in inoculation; this result will be the plaque-forming units (PFUs) per bird dose (assuming the volume of 1 bird dose is 0.2 ml)

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\*125 (dilution factor) divided by 5 (number of doses per ml) equals 25.

\*\*250 (dilution factor) divided by 5 (number of doses per ml) equals 50.

**Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3**

### **6.3 Retests**

Conduct retests as required by the Code of Federal Regulations, Title 9 (9CFR), Part 113.8 (b) and requirements of minimum release in firm's current Outline of Production, Part V.

### **6.4 Evaluation of test results**

**6.4.1** The 9 CFR 113.8(b) defines the criteria for a satisfactory/unsatisfactory serial.

**6.4.2** The firm's requirements of minimum release/stability titers for each Marek's vaccine are listed in the current Outline of Production, Part V, for the specific product code.

## **7. Report of test results**

Titers are reported out as PFUs per bird dose.

## **8. Summary of revisions**

This document was revised to clarify the practices currently in use in the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- The title of the SAM has been revised to reflect changes in the nomenclature for the organism formally known as Herpesvirus of Turkeys and Chicken Herpesvirus to Marek's Disease Vaccine (as outlined in a Veterinary Biologics Notice dated June 12, 1996) with multiple serotypes that have been identified and marketed.
- **2.2.15(1)** The trypsin solution (0.25%) has been revised for clarity.
- **2.2.15(2)** and **2.2.15(3)** HEPES has been added to the mediums and "heat-inactivated" has been changed to "gamma-irradiated" for the Fetal Bovine Serum.

Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3

- **3.** This section on cell cultures has been added for clarity.
- **Appendix I** This section has been added for clarity of counting cells in suspension with a Neubauer hemacytometer and formulating cell suspensions.

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Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3

## 9. Appendix

### Appendix I

#### Counting Cells in Suspension with a Neubauer Hemacytometer and Formulating Cell Suspensions

The protocol in this appendix describes a method to count cells in suspension with the use of a 1/10 mm deep Neubauer hemacytometer and microscope. It also explains how to formulate cell suspensions for specific concentrations and volumes.

#### 1. Materials

##### Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

1. Hemacytometer (1/10 mm deep Neubauer hemacytometer)
2. Incandescent microscope (capable of 100X magnification)
3. Hand Tally Counter

##### Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

1. Trypan Blue (0.4% dissolved in 0.85% normal saline)
2. Snap cap tube, 6 ml, 12 X 75-mm
3. Disposable plastic pipettes, 1-, 5-, 10-, and 25-ml
4. Pipette (LabSystems, Model Finn timer Digital 40-200 ③1)
5. Disposable pipette tips

Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3

## 2. Counting cells with a hemacytometer

In a 6-ml snap cap tube, dilute 0.5 ml of the cell suspension to be counted with 1.0 ml of 0.4% Trypan Blue solution. Replace the cap on the tube and mix the suspension by vortexing. Place the cover slip over the counting grid on the hemacytometer. While the cells are still suspended, quickly load the chamber of the hemacytometer with this preparation using a pipette. Fill the chamber with enough of the preparation to completely cover the grid under the cover slip. Overfilling can cause erroneous counts. The total volume of cell suspension in 10 1-mm squares (see numbered squares) under the cover slip is equal to 1 cubic mm or 1  $\mu$ l.

1		2
	5	
4		3

  

6		7
	1 0	
9		8

Figure 1

Let the preparation stand for approximately 1 minute to allow the cells to settle onto the bottom of the counting chamber. Examine the grid with a microscope under 100X magnification. Check for the even distribution of cells. (Any irregularity of distribution will cause erroneous results.) Count the live cells (dead cells stain blue while live cells remain translucent). Count only those live cells that fall on the top line, the left line, or within each numbered 1-mm square (1-10) in the grid (**Figure 1**). Keep track of the total number of cells counted for the 10 squares with a Hand Tally Counter.

Calculate the number of cells per ml in the initial suspension by multiplying the total cell count by the dilution in Trypan Blue (3) and by the conversion factor from cubic mm ( $\mu$ l) to ml (1000). See example below.

**Supplemental Assay Method for Titration of Monovalent, Cell Associated Marek's  
Disease Vaccines of Serotypes 1, 2, or 3**

Example: number of cells in 10 squares= 385  
1:3 cell dilution=  $\frac{x}{3}$   
1155  
cubic mm/ml  $\times 1000$   
number of cells per ml= 1155000

**4. Formulating cell suspensions**

1. Calculate the Dilution Factor (DF) by dividing the concentration of cells/ml in the initial suspension by the desired final concentration of cells/ml.

Example:

If the concentration of cells in the initial suspension is 1155000 cells/ml and the desired final concentration is 350000 cells/ml, then:

$$DF = 1155000 \text{ cells/ml} \div 350000 \text{ cells/ml} = 3.3$$

2. Divide the desired final volume of cells by the DF. The result of this division will tell you the volume of the initial cell suspension you will need to make the final desired volume of cells.

Example:

If the final desired volume of cells is 375 ml, then:

$$375 \text{ ml} / 3.3 \text{ (DF)} = 113.6 \text{ ml}$$

This is the volume of the initial cell suspension needed to make the final volume of cells.

3. Take the amount of the initial cell suspension and add sufficient media to bring it to the final volume of cells desired.

Example:

Add 261 ml (375 ml - 114 ml) of media to 114 ml of the initial cell suspension (1155000 cells/ml) to get 375 ml of the desired 350000 cells/ml suspension.